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Phage display vectors for in vivo recombination of immunoglobulin heavy and light chain genes to make large combinatorial libraries

(*lox*-Cre site-specific recombination; single-chain Fv fragment; *Escherichia coli* expression; human selectins)

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SUMMARY

New phage display vectors for in vivo recombination of immunoglobulin (Ig) heavy (V_H) and light (V_L) chain variable genes, to make single-chain Fv fragments (scFv), were constructed. The V_H and V_L genes of monoclonal antibody (mAb) EP-5C7, which binds to both human E- and P-selectin, were cloned into a pUC19-derived plasmid vector, pCW93, and a pACYC184-derived phagemid vector, pCW99, respectively. Upon induction of Cre recombinase (phage P1 recombinase), the V_H and V_L genes were efficiently recombined into the same plasmid via the two *loxP* sites (phage P1 recombination sites), one located downstream from a V_H gene in pCW93 and another upstream from a V_L gene in pCW99. In the resulting phagemid, the *loxP* sequence also encodes a polypeptide linker connecting the V_H and V_L domains to form a scFv of EP-5C7. Whether expressed on the phage surface or as a soluble form, the EP-5C7 scFv showed specific binding to human E- and P-selectin. This phagemid vector system provides a way to recombine V_H and V_L gene libraries efficiently in vivo to make extremely large Ig combinatorial libraries.

INTRODUCTION

M13 phage surface display vectors (Smith, 1985) have been used successfully for isolation and engineering of mAb (for review, see Burton et al., 1994; Winter et al., 1994). Antigen binding sites of Ab are displayed as Fab or scFv on the surface of filamentous phage (McCaffetry et al., 1990; Barbas et al., 1991; Chang et al., 1991). Phage with a desired antigen specificity can be isolated by binding to and elution from target antigens. Phage display vectors have also been used to improve the binding affinities of various antibodies (Burton et al., 1994; Winter et al., 1994).

In theory, the larger the Ab repertoire in a phage display library, the higher the chance to isolate rare mAb with desired properties. However, in practice, the library size is limited by the transformation efficiency of *E. coli*. It is not feasible to make a phage Ab library larger than 10^9 by transformation of *E. coli*. To overcome this limitation, Waterhouse et al. (1993) used the *lox*-Cre site-specific recombination system (Sternberg et al., 1981) to develop a phage vector in which V_H and V_L genes are recombined in vivo and expressed as Fab on the phage surface.

In this paper, we report a new surface display plasmid vector system which allows efficient recombination

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Abbreviations: aa, amino acid(s); Ab, antibody(ies); *araBpo*, promoter-operator of *araB*; Ap, ampicillin; Cκ, constant region of κ light chain; Cm, chloramphenicol; Cre recombinase, phage P1 recombinase (product of gene *cre*); Δcp3, C-terminal domain of M13 minor coat protein III; ELISA, enzyme-linked immunosorbent assay; Fab, Fab fragment(s) of Ab; f1 *ori*, replication origin of phage f1; Ig, immunoglobulin; IPTG, isopropyl-β-D-thiogalactopyranoside; *lacZpo*, promoter-operator of *lacZ*; *loxP*, phage P1 recombination site; mAb, monoclonal Ab; myc peptide, short polypeptide derived from human c-myc protein; nt, nucleotide(s); scFv, single-chain Fv fragment(s) of Ab; Tc, tetracyclin; tu, transforming unit(s); V_H and V_L, Ig heavy and light chain variable, respectively; [], denotes plasmid-carrying state.

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between V_H and V_L genes from separate plasmids in *E. coli* using the *lox*-Cre system to form a gene on one plasmid to produce scFv. Using this system, the size of the Ab repertoire is limited essentially only by the number of *E. coli* used for in vivo recombination of V_H and V_L genes, and therefore it is possible to construct extremely large Ig combinatorial libraries.

EXPERIMENTAL AND DISCUSSION

(a) Phage display vectors with the *lox*-Cre recombination system

Three plasmids, pCW93 for cloning of V_H genes, pCW99 for cloning of V_L genes and pCre for production of Cre recombinase (Sternberg et al., 1981), were constructed for in vivo recombination between V_H and V_L genes to form a gene to produce scFv (Fig. 1). These three plasmids are compatible with each other and can coexist in the same host *E. coli*.

The V_H gene of humanized mAb EP-5C7, which binds to both human E- and P-selectin (Berg et al., 1995; X. Y. He and M. S. Co, unpublished), was cloned as a *NcoI-NheI* fragment into pCW93, a derivative of pUC19 (Yanisch-Perron et al., 1985), to make pCW93/H (Fig. 1). The V_H gene was linked to the *pelB* signal peptide (Lei et al., 1987) and placed downstream from *lacZpo*. The *loxP* site was located downstream from the V_H gene. In addition, the *loxP*511 site (Hoess et al., 1986), which has one nt substitution in the spacer region of the *loxP* site, was located further downstream.

The V_L gene of humanized EP-5C7 was cloned as a *SacI-BglII* fragment into the phagemid vector pCW99, a derivative of pACYC184 (Chang et al., 1978), to make pCW99/L (Fig. 1). This vector carries the constant region of human κ light chain-encoding gene (C_K), a DNA segment encoding a short polypeptide derived from human c-Myc protein (Myc peptide) (Evan et al., 1985), the C-terminal domain of M13 minor coat protein III (*Δcp3*), and the replication origin of bacteriophage f1 (f1 *ori*) (Akamatsu et al., 1993). The *loxP* site was located upstream from the V_L gene, and the *loxP*511 site downstream from f1 *ori*.

The gene for Cre recombinase of phage P1 was cloned downstream from *araBpo* (Schleif, 1987) in the pSC101-derived plasmid pLG339 (Stoker et al., 1982) (Fig. 1). In *E. coli* harboring pCre, production of Cre recombinase can be induced by arabinose. Cre recombinase catalyzes site-specific recombination between two *loxP* sites (Sternberg et al., 1981). Recombination also occurs efficiently between two *loxP*511 sites; however, it does not occur between the *loxP* and *loxP*511 sites (Hoess et al., 1986).

(b) Site-specific recombination between V_H and V_L genes

In *E. coli* harboring pCW93/H, pCW99/L and pCre (Fig. 1), site-specific recombination between the V_H and V_L genes mediated by the *lox*-Cre system can be induced by adding arabinose in culture media. When pCW93/H and pCW99/L are recombined via the *loxP* sites, plasmid A in Fig. 1 is formed. When the *loxP*511 sites are used, plasmid B is formed (Fig. 1). However, these two plasmids appear to be unstable in the presence of Cre recombinase because the two *loxP* (and *loxP*511) sites on the same plasmid are closely located and are therefore preferentially used for further recombination. This series of recombination results in reversion to the original two plasmids pCW93/H and pCW99/L or generation of two new plasmids C and D (Fig. 1), depending on which of the *loxP* or *loxP*511 sites are used in intermediate plasmids A and B.

In plasmid C, the *loxP* sequence also serves the function of encoding a part of the peptide linker connecting V_H and V_L domains to form scFv, which is further connected to human C_K and then to Δcp3 for display on the phage surface (Fig. 2). In addition, an amber stop codon is located just upstream from Δcp3. Gln is inserted at this amber codon in *supE* mutants such as TG1ΔrecA (*supE*, *hsdΔ5*, *thi*, Δ(*lac-proAB*), Δ*recA* [F', *proAB*$^+$, *lacI*^{q}, *lacZ*ΔM15]) (Akamatsu et al., 1993); however, when *lacZpo* is induced by IPTG, a soluble form of scFv connected to C_K is produced even in TG1ΔrecA due to the low level of amber suppressor (Akamatsu et al., 1993).

(c) Efficiency of recombination

TG1ΔrecA cells harboring pCW99/L were superinfected by VCSM13 helper phage (Stratagene, La Jolla, CA) to package the phagemid DNA into M13 phage particles. Then, TG1ΔrecA cells harboring pCW93/H and pCre were infected by phage containing pCW99/L DNA and VCSM13 helper phage, and cultured overnight with or without arabinose. Only the plasmids carrying f1 *ori*, such as pCW99/L, plasmids A, B and C (Fig. 1), are packaged into phage particles. The titer of phage containing pCW99/L and plasmid C DNA can be measured as Cm^R and Ap^R transforming units (tu), respectively. Plasmids A and B can be measured as Cm^R-and-Ap^R tu.

In the absence of arabinose, Cm^R tu was 1.1×10^{10}/ml while Ap^R tu and Cm^R-and-Ap^R tu were both less than 10^6/ml. On the other hand, in the presence of arabinose, nearly the same number of Cm^R and Ap^R colonies were obtained; Cm^R tu was 7.3×10^9/ml and Ap^R tu was 5.9×10^9/ml. Cm^R-and-Ap^R tu was 1.0×10^8/ml. DNA analysis showed that all of the twelve randomly picked Ap^R colonies contained plasmid C (data not shown). These results indicate that the formation of plasmid C by *lox*-Cre site-specific recombination occurs very efficiently.

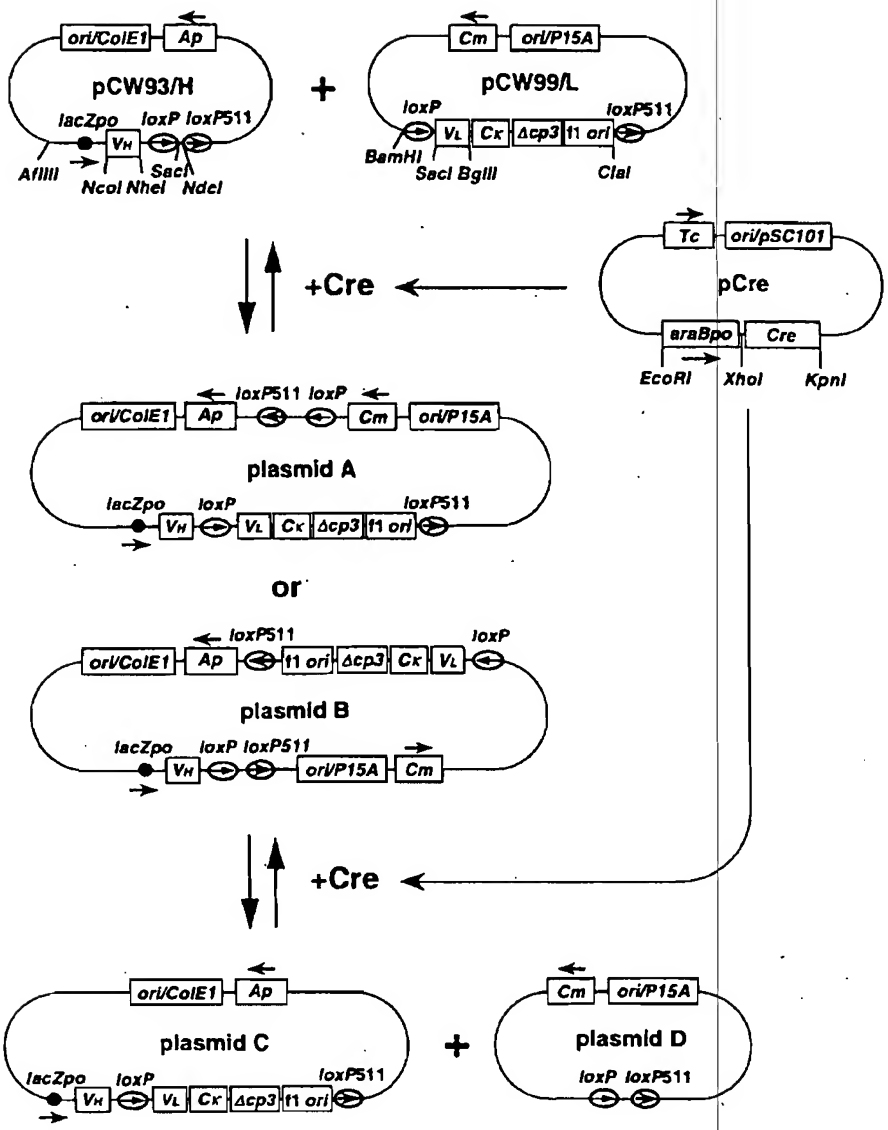

Fig. 1. Structures of plasmids for in vivo recombination of V_H and V_L genes. To construct pCW93 and pCW99, pScUAGΔcp3 (Akamatsu et al., 1993) was first modified to introduce a *Nco*I site, the *loxP* sequence and the myc peptide-coding sequence as shown in Fig. 2. The *Afl*III-*Sac*I fragment of the resulting plasmid, pNT3148, containing the cloning sites for V_H genes and the *loxP* site was inserted between the corresponding sites of pUC19. The *loxP*511 site 5'-ATAACTTCGTATAGTATACATTATACGAAGTTAT; the nt mutated from the wild-type *loxP* is underlined) was inserted at the *Nde*I site (pCW93). The V_H gene of humanized anti-human E/P-selectin Ab EP-5C7 was inserted between the *Nco*I and *Nhe*I sites to make pCW93/H. To construct pCW99, the *Bam*HI-*Cla*I fragment of pNT3148 containing the *loxP* site, the cloning sites for V_L genes for human Cκ, myc peptide, Δcp3 and f1 *ori* was inserted between the *Hin*cII (nt 3211; Rose, 1988) and *Cla*I sites of pACYC184 after converting the *Hin*cII site to a *Bam*HI site, and then the *loxP*511 site was inserted into the *Cla*I site. The V_L gene of humanized EP-5C7 was inserted between the *Sac*I and *Bgl*II sites to make pCW99/L. To construct pCre, the *Pvu*II site of pLG339, a derivative of pSC101, was first converted to a *Kpn*I site (pLG339-K). Then, the *Eco*RI-*Xho*I fragment of pAraCPB containing the *araB* promoter and the *araC* gene (M. Imai, unpublished) and the *Xho*I-*Kpn*I fragment containing the *cre* gene of phage P1 obtained by polymerase chain reaction (Mullis et al., 1986) were inserted between the *Eco*RI and *Kpn*I sites of pLG339-K (pCre). The *ori*'s of pUC19, pACYC184 and pSC101 are shown as *ori/ColE1*, *ori/P15A* and *ori/pSC101*, respectively. Genes for Ap, Cm and Tc resistance are shown. Arrows show direction of transcription. In TG1ΔrecA cells harboring pCW93/H, pCW99/L and pCre, when the production of Cre recombinase is induced by arabinose, pCW93/H and pCW99/L recombine to plasmid A via the *loxP* sites or to plasmid B via the *loxP*511 sites. Plasmids A and B are further recombined via the *loxP* or *loxP*511 sites on the same plasmid to make the original two plasmids pCW93/H and pCW99/L, or two new plasmids C and D.

In addition, the recombination event was tightly regulated by the expression of Cre recombinase under the control of *araBpo*.

(d) Functional analysis of EP-5C7 scFv

In order to confirm that scFv expressed from plasmid C is functional, TG1ΔrecA cells harboring plasmid C

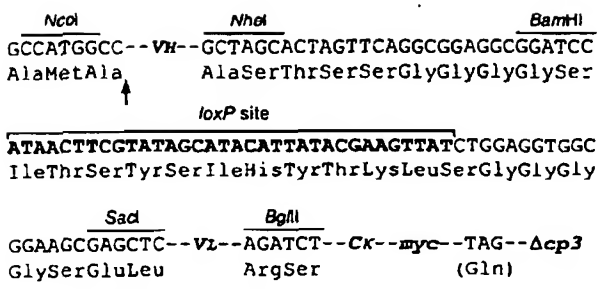

Fig. 2. The aa sequence of the polypeptide linker connecting VH and VL domains. The nt and aa sequences surrounding the *loxP* site in the scFv-producing plasmid C are shown. The *loxP* site is shown in boldface. The arrow indicates the cleavage site of the leader peptide. Relevant restriction sites are shown above their recognition sequences. The TAG amber stop codon located upstream from Δcp3 is translated to Gln in the presence of the *supE* gene.

were grown in the presence of IPTG and culture supernatants containing soluble scFv were used for ELISA as described previously (Akamatsu et al., 1993). Wells of an ELISA plate were coated with purified human E-, P- or L-selectin. After incubation with IPTG-induced culture supernatant, bound scFv was detected by goat anti-human κ chain Ab conjugated with peroxidase (Biosource, Camarillo, CA). As was observed with mAb EP-5C7 (Berg et al., 1995), soluble EP-5C7 scFv bound to E- and P-selectin, but not to structurally related L-selectin. In addition, specific binding of phage particles with surface-displayed EP-5C7 scFv to E- and P-selectin was observed by ELISA using anti-M13 phage Ab conjugated with peroxidase (Pharmacia, Piscataway, NJ) for detection of bound phage. Similarly, when the VH and VL domains of monoclonal anti-DNA Ab K4-2 (N.T., unpublished) were connected by the same polypeptide linker shown in Fig. 2, soluble K4-2 scFv showed specific binding to DNA. Thus, the aa sequence encoded by the *loxP* sequence provided a flexible polypeptide linker which allows VH and VL domains to form a functional antigen-binding site.

(e) Conclusions

(*1*) Using the *lox*-Cre site-specific recombination system, we have developed phagemid vectors which allow efficient recombination between *V*H and *V*L genes from separate plasmids to form scFv-producing phagemid in *E. coli*. Recombined *V*H and *V*L genes can be expressed as surface-displayed scFv on M13 phage or as a soluble scFv by IPTG induction.

(*2*) The efficiency of recombination between *V*H and *V*L genes is very high. Nearly half of the phage particles rescued from *E. coli* after *lox*-Cre-mediated recombination contained the desired scFv-producing phagemid.

(*3*) The *loxP* sequence is used to encode a part of the polypeptide linker connecting VH and VL domains. The polypeptide linker was shown to not disturb association of VH and VL domains in forming a functional antigen-binding site.

(*4*) Unlike the method by Waterhouse et al. (1993) which requires infection of phage P1, Cre recombinase is supplied from a plasmid which coexists with *V*H- and *V*L-carrying plasmids in our system. In addition, production of Cre recombinase is controlled by *araBpo*.

(*5*) Our surface display phagemid vector system can be used to recombine *V*H and *V*L gene libraries to construct very large Ig combinatorial libraries. The size of antibody repertoire depends essentially on the number of *E. coli* used for in vivo recombination of *V*H and *V*L genes. It is therefore possible to make a combinatorial library with the size of 10^{12} by using several liters of *E. coli* culture. Such large libraries may allow the isolation of rare mAb with desired binding properties.

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